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(54) Title: A METHOD OF IDENTIFYING LIGANDS AND ANTAGONISTS OF LIGANDS

(57) Abstract

The present invention relates, in general, to a method of identifying ligands and antagonists of ligands. In particular, the present invention relates to a method of identifying ligands and antagonists of ligands which bind to cloned G_S- or G_I-coupled receptors. The present invention also relates to a cell that comprises a recombinant cyclic AMP sensitive reporter construct.

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A METHOD OF IDENTIFYING LIGANDS AND ANTAGONISTS OF LIGANDS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates, in general, to a method of identifying ligands and antagonists of ligands. In particular, the present invention relates to a method of identifying ligands and antagonists of ligands which bind to cloned G_i- or G_i-coupled receptors. The present invention also relates to a cell comprising a recombinant cyclic AMP sensitive reporter construct.

Background Information

In the last several years, numerous cDNAs or genes for hormones and neurotransmitters that couple to G-proteins have been cloned. With only two exceptions - the receptors for insulin-like growth factor II and glutamic acid - these G-coupled receptors are rather similar, and they are thought to comprise a large "superfamily" of proteins. By analogy to bacterial rhodopsin, the members of this superfamily are thought to contain seven membrane-spanning domains and have a number of highly conserved amino acids. As a consequence, oligonucleotide probes directed at shared domains of cloned receptors have been used at low stringency to screen cDNA libraries for novel receptor candidates. More recently, primers directed at conserved domains of these receptors have been used in the polymerase chain reaction to clone additional candidates. The cDNAs obtained as described above, which encode "orphan receptors" for which the endogenous ligand is not

known, must be expressed and the ligands which th r c ptors bind must be identifi d. This is not as straightf rward as it s ems at first glanc . Scr ening with radiolab ll d ligands is xpensiv . It r quir s many (unstabl) ligands and large amounts of transfected cells. Functional screens, on the other hand, are hampered by the fact that one cannot guess from the structure of a receptor which second messenger system it activates. Some G-protein coupled receptors activate phospholipase C or phospholipase A_2 and increase inositol phosphates and arachidonic acid, respectively. Others activate (G_s-coupled) or inhibit the activation (G_i-coupled) of adenylate cyclase, increasing or decreasing the level of cyclic AMP in cells. The present invention provides simple and rapid methods for discovering the agonists which act on orphan G_s- or G_i-coupled receptors.

The assay that has been developed employs a novel mouse L cell line, LVIP2.OZc. These L cells contain a stably integrated fusion gene pLVIP2.OZ plasmid (Riabowol, K.T. et al., (1988) Nature 336:83-86), consisting of the Escherichia coli lac Z gene under the transcriptional control of 2kb fragment derived from the vasoactive intestinal polypeptide (VIP) gene. This DNA segment includes the VIP promoter and a cyclic AMP responsive enhancer element (CRE). Forskolin increases cellular cyclic AMP levels and thus can be used to induce the ß-galactosidase enzyme in the LVIP2.OZc. reporter cells. After the cells are lysed, the enzyme activity can be detected by addition of a chromogenic substrate, o-nitrophenyl ß-D-galactopyranoside (ONPG). The product of the reaction, o-nitrophenol, is yellow in color and can be seen with the naked eye or measured spectrophotometrically at a wavelength of 405nm.

The assay can be performed in 96 well tissue culture plates and a commercially available plate reader can be used to measure the levels of o-nitrophenol and record the results.

Transfection of cells with a putative G_i-coupled receptor allows one to induce the enzyme by adding the appropriate ligand. For example, cells transfected with a β_2-adrenergic receptor cDNA increase their β-galactosidase levels in response to isoproterenol. G_i-coupled receptors, on the other hand, inhibit the activation of adenylate cyclase. In the presence of the appropriate ligands the dopamine D_2, muscarinic cholinergic m_2, or cannabinoid transfected receptors significantly reduce the forskolin-stimulated increase in β-galactosidase normally seen in LVIP2.OZc cells. Thus, LVIP.OZc cells provide a rapid, convenient and semi-automated system in which a large number of putative ligands may be screened for binding to an "orphan receptor" transiently expressed in these cells.

SUMMARY OF THE INVENTION

It is a general object of this invention to provide a method of identifying ligands and antagonists of ligands.

It is a specific object of this invention to provide a method of identifying ligands which bind to G-protein coupled receptors.

It is another object of this invention to provide a method of identifying an antagonist of a ligand wherein the ligand binds to a G-protein coupled receptor.

It is a further object of this invention to provide a cell that comprises a recombinant cyclic AMP sensitive reporter construct.

Further objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Detection of β-galactosidase of color development of o-nitrophenol. LVIP2.OZc cells were exposed for 8 hours to control medium (containing 0.5mM IBMX) (□), or control medium containing 0.5μM forskolin (•), or control medium containing 1μM (■) or 10 NECA (▲). After cell lysis and addition of the substrate ONPG, enzyme activity was measured as optical density at 405nm at various time intervals. Data are mean ± S.D. taken from one experiment that is representative of several.

Figure 2. Induction of β-galactosidase as a response to length of ligand exposure. LVIP2.OZc cells were incubated for 1, 2, 4, 6, 8, 12, 15 and 18 hours in control medium (containing 0.5mM IBMX) (□), or control medium containing 0.5μM forskolin (•), or control medium containing 1μM NECA (■), or control medium containing 1μM PGE_2 (▲). Data are mean ± S.D. from one representative experiment.

Figure 3. Induction of β-galactosidase as a response to different doses of ligands. LVIP2.OZc cells were treated for 8 hours with control medium (containing 0.5mM IBMX), and 0.1 or 0.2 or 0.5 or 1μM forskolin (•), control medium and 0.1 or 0.5 or 1 or 10μM NECA (■), control medium containing 0.1 or 0.5 or 1 or 10μM PGE_2 (▲). Data are mean ± S.D. from a representative experiment.

Figur 4. A) Induction of ß-galact sidase as a dose-r spons f r $ß_2$-adrenergic r c pt r DNA transf ct d LVIP2.OZc c lls to isoproter n l. Cells w re incubat d for 8 h urs with medium containing 0.5mM IBMX, 100µM ascorbic acid and $5x10^{-12}$ to 10^{-4} M isoproterenol. Data are mean ± S.D. from a representative experiment. B) Induction of ß-galactosidase as a time-response for $ß_2$-adrenergic receptor DNA transfected LVIP2.OZc cells to isoproterenol. Cells were exposed for 2, 4, 6, 8, 12 and 18 hours to medium containing 0.5mM IBMX, 100µM ascorbic acid ± 1µM isoproterenol. Data are mean ± S.D. from a representative experiment.

Figure 5. Inhibition of forskolin-mediated induction of ß-galactosidase by aldosterone secretion inhibitory factor during different exposure times. LVIP2.OZc cells were exposed for 1, 2, 4, 6, 8, 15, or 18 hours to control medium (containing 0.5mM IBMX and 20ug/ml Bacitracin) (□), or control medium containing 0.5µM forskolin + 1 µM ASIF (O), or - ASIF (•). Data are mean ± S.D. from a representative experiment.

Figure 6. A) Inhibition of forskolin-mediated induction of ß-galactosidase in dopamine receptor DNA transfected LVIP2.OZc cells as a dose-response to different concentrations of dopamine or quinpirole. Cells were exposed for 6 hours to medium containing 0.5mM IBMX and 100µM ascorbic acid and 0.5µM forskolin ± 1 or 10µM dopamine hydrochloride or 1, 10, 100µM quinpirole hydrochloride. Data are mean ± S.D. from one representative experiment. B) Inhibition of forskolin-mediated induction of ß-galactosidase in

cannabinoid receptor DNA transfected LVIP2.OZc cells by CP55940. Cells were exposed for 6 hours to medium containing 0.5mM IBMX, 0.5mM BSA and 0.5μM forskolin ± 1μM CP55940. Data are mean ± S.D. from one representative experiment. C) Inhibition for forskolin-mediated induction of β-galactosidase in human muscarinic acetylcholine (hm_2) receptor DNA transfected LVIP2.OZc cells by carbachol. Cells were exposed for 6 hours to medium containing 0.5mM IBMX and 0.5μM forskolin ± mM carbachol. Data are mean ± S.D. from one representative experiment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a fast, simple method to identify (1) ligands which bind to a G-protein coupled receptor and (2) antagonists of these ligands.

In one embodiment, the method comprises: i) expressing a G_i-coupled receptor gene in a cell wherein the cell contains a cyclic AMP sensitive reporter construct; ii) adding a ligand to the cell; and iii) assaying for the amount of cyclic AMP.

In general, the construct comprises a cyclic AMP responsive regulatory element operably linked to a reporter gene. In a preferred embodiment, the cyclic AMP sensitive reporter construct comprises a cyclic AMP responsive enhancer element operably linked to a promoter and a reporter gene. Suitable cyclic AMP responsible enhancer (CRE) elements are well known in the art. They include but are not limited to the CRE located on the 2 kb DNA fragment from the vasoactive intestinal polypeptide (VIP) gene. Suitable promoters are also well known in the art.

They include but are not limited to the VIP promoter described herein. Further, suitable reporter genes are also well known in the art. They include but are not limited to: the Lac Z gene and the luciferase gene. Specifically, the reporter construct used may be pLVIP2.OZ.

The G-coupled receptor gene expressed in the cell may be stably incorporated into the genome of the cell, may be transiently transfected into the cell, or may be endogenous to the cell. Examples of G-coupled receptors include: the human β_2-adrenergic receptor; the human m_2 muscarinic acetylcholine receptor; the rat cannabinoid receptor; the dopamine D_{2L} receptor; VIP; secretin; vasopressin; oxytocin; serotonin; α-adrenergic; metabolic glutamate; and IL-8 receptors.

Suitable host cells, if appropriate vectors are used, include both lower eucaryotes (for example, yeast) and higher eucaryotes (for example, mammalian cells - specifically, mouse cells). Preferably, the assay uses a mouse L cell line (for example - LVIP2.OZc, ATCC accession no. CRL 10871) which contains a cyclic AMP responsive reporter construct (for example - pLVIP2.OZ).

The methods of assaying for the expression of the reporter genes are well known in the art. In a preferred embodiment, the assaying step comprises adding a chromogenic substrate and assaying for a change in that substrate. One preferred chromogenic compound is o-nitrophenyl ß-D-galactopyranoside.

In a further embodiment, the present invention relates to a method of identifying an antagonist of a ligand wherein the ligand binds to a G-protein coupled receptor. The method comprises: i) expressing a G_s-coupled receptor gene or cDNA in a cell wherein the cell contains a

cyclic AMP sensitive reporter construct; ii) adding a ligand and antagonist to the cell; and iii) assaying for the amount of cyclic AMP. The cyclic AMP reporters, reporter genes, constructs, and cells described-above can be used to identify antagonists.

In a further embodiment, the present invention relates to a method of identifying a ligand that binds to a receptor which inhibits stimulation of adenylyl cyclase. The method comprises: i) expressing a G_i-coupled receptor gene of cDNA in a cell wherein the cell contains a cyclic AMP sensitive reporter construct; ii) adding forskolin and candidate ligands; and iii) assaying for the amount of cyclic AMP.

In a further embodiment, the present invention relates to a method of identifying an antagonist of a ligand that binds to a receptor which inhibits stimulation of adenylyl cyclase. The method comprises: i) expressing a G_i-coupled receptor or cDNA in a cell wherein the cell contains forskolin and a specific ligand and candidate antagonists; and iii) assaying for the amount of cyclic AMP, looking for the inhibition of agonist induced reduction of forskolin activity.

The method described herein can be used to screen large numbers of ligands: for example, as many as 22 ligands can be analyzed in quadruplicate in a single 96 well plate. The stimulation or inhibition of stimulation is easy to recognize, and positive results can quickly be confirmed.

The present invention is described in further detail in the following non-limiting Examples.

EXAMPLES

The following protocols and experimental details are referenced in the Examples that follow:

Materials. Unless otherwise indicated, chemicals were obtained from SIGMA, culture media from Whittaker Bioproducts, ASIF (Aldosterone Secretion Inhibitory Factor) [Bovine] was purchased from PENINSULA LABORATORIES, ONPG (o-NITROPHENYL-ß-D-galactopyranoside) was obtained from RESEARCH ORGANICS, (-)-Propanolol hydrochloride, Dopamine hydrochloride, (-)-Quinpirole hydrochloride were obtained from RBI, CP55940 was generously provided by PFIZER, INC.

Stable cell line and cell culture. Mouse Ltk- cells ($3x10^6$ per 10cm dish) were transfected using the calcium phosphate co-precipitation method with the pVIP2.OZ plasmid (20 µg) containing 2 kb of 5'-flanking sequences form the human VIP gene fused to the E. coli lac Z gene (Riabowol et al. (1988) Nature 336:83-86). Co-transfection with 4 µg phyg (Sugden et al. (1985) Mol. Cell. Biol. 5:410), a plasmid encoding hygromycin B phosphotransferase, and selection in hygromycin provided a dominant selectable marker. Hygromycin-resistant Ltk$^-$ clones were screened for induction of ß-galactosidase after addition of forskolin (10 µM) and IBMX (0.5 µM). One clone, LVIP2.OZc, was chosen for use in these experiments. LVIP.OZc cells, stably transfected with the pVIP2.OZ plasmid were maintained in Dulbecco modified Eagle medium (DMEM), supplemented with 2.5% fetal calf serum, 7.5% newborn calf serum, 25 mg/ml hygromycin, penicillin and streptomycin in a 7% CO_2 37° Forma

incubator. Cells were trypsinized twice weekly with 1 ml 0.5% Trypsin and .53 mM EDTA.4Na /10 cm dish and split at a ratio of 1:10 and grown in 10 ml medium over several months. The phenotype of the cells seems stable.

Plasmid DNA. Plasmid DNA was prepared by the lysozyme-Triton procedure (Katz, L. et al., (1973) J. Bacteriol. 114:577-591) as modified for the transfection protocol of Chen and Okayama ((1987) Mol. Cell. Biol. 7:2745-2752). The following receptor cDNAs were introduced into the pcD plasmid (Okayama, H. et al., (1983) Mol. Cell. Biol. 3:280-289): the human β_2-adrenergic receptor (Kobilka, B.K. et al., (1987) PNAS 84:46-50), the human m_2 muscarinic acetylcholine receptor (Bonner, T.I. et al., (1987) Science 237:527-531), the rat cannabinoid receptor (Matsuda, L.A. et al., (1990) Nature 346:561-564). The dopamine D_2 receptor (Monsma, F.J. et al., (1989) Nature 342:926-929) was subcloned into the pRc/RSV vector (INVITROGEN, eukaryotic vector).

Transfection of cells. Cells were transfected by the calcium phosphate transfection protocol of Chen and Okayama. For each plasmid DNA preparation, the amount of DNA required for formation of an optimum calcium phosphate-DNA precipitate varies; thus, a dose response study is desirable. To set up conditions to assay G_i-coupled receptors, cells were transfected with various amounts (5 to 30μg) of pcD plasmid DNA containing the human β_2-adrenergic receptor cDNA/10-cm plate. If smaller or larger dishes were used, the amount of DNA was adjusted proportionally. The precipitate formed with 5μg of DNA usually was very coarse; with 25 to 30μg of

DNA th pr cipitate was usually v ry fi wh observ d with a microsc pe at low magnification (40x). Eight to 20μg gav a punctuat pr cipitat which s ems t be taken up by th cells efficiently. Cells were trypziniz d 48 hours after transfection, pelleted, resuspended in fresh medium, seeded into 96 well microtiter plates (flat bottom) at 5-10 x 10^4 cells/well in 100μl medium, and incubated for an additional 24 hours. Then 100μl of medium containing isoproterenol (2μM), ascorbic acid (200μM) and isobutyl methyl xanthine (1mM) (IBMX, a phosphodiesterase inhibitor) was added to each well. Adding medium rather than replacing the medium in each well gave more reproducible results. One hundred microliters of medium containing IBMX/ascorbic acid or forskolin (1μM)/IBMX/ascorbic acid were added to control wells. To establish conditions to assay G_i-coupled receptors, cells were transfected with the dopamine D_2 plasmid DNA as described above, seeded into 96 well microtiter plates in 100μl medium and the appropriate ligands in 100μl medium were tested for the inhibition of forskolin-stimulated ß-galactosidase activity. Each set of conditions was carried out in quadruplicate. After an eight hour exposure to ligand the cells were washed with 200μl phosphate buffered saline and ß-galactosidase activity was assayed. The plates can be stored at -20°C for 24-72 hours before performing the ß-galactosidase assay.

ß-Galactosidase microassay. ß-galactosidase activity was measured with a microplate reader (Molecular Devices, Palo Alto). The assay is a modified version of the o-nitrophenyl ß-D-galactoside (ONPG)-based assays of Perrin ((1963)

Ann. N. Y. Acad. Sci. 81:6349-6353) and Norton and Coffin ((1985) Mol. Cell. Biol. 5:281:290). The modifications include the method of cell lysis and conditions of assay incubation as outlined below. Suppressing endogenous galactosidase activity and stabilizing the E. coli enzyme are critical for the success of the assay.

All the steps in the microassay were carried out at room temperature. The assay buffer is composed of 100 mM sodium phosphate, 2mM $MgSO_4$, 0.1mM $MnCl_2$, pH 8.0. The adherent cells were washed with PBS, the plate drained and 25µl/well of hypotonic lysis buffer added to each well, (dilute 1 part buffer with 9 parts water). Ten minutes later 100µl of assay buffer containing 0.5% (v/v) TritonX-100 and 40mM ß-mercaptoethanol were added. Care should be taken to avoid foaming. After an additional 10 minutes, 24µl of ONPG (4mg/ml in assay buffer; prepared daily) was added, and the optical density per well at 405nm was determined with a plate reader. Readings were taken through 1.5 OD at 405nm, within the linear portion of the color development. The initial rates, expressed as mOD/minute, are linearly related to ß-galactosidase enzyme concentration over an enzyme concentration range of 0.1 to 100mU (one unit hydrolyzes one micromole of ONPG per minute at 25 degrees, pH 7.5).

A detailed description of the screening protocol is provided in Table 1.

EXAMPLE 1

Detection of β-D-galactosidase in LVIP2.OZc cells

In preliminary experiments, forskolin (5μM) and IBMX (0.5mM) was used to increase cyclic AMP levels. The cells were treated with the drugs for 3, 6, or 18 hours, fixed with 2% formaldehyde and 0.2% glutaraldehyde for 10 minutes at room temperature, and exposed to the chromogenic substrate, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 0.2 mg/ml) for 2 hours at 37°C. More than 90% of the cells treated with drugs for 6 to 18 hours developed a blue color. Only a few cells treated with IBMX alone turned blue. Next, the cells were transfected with human β_2-adrenergic receptor cDNA in the pcD expression vector. Seventy-two hours after the DNA was added to the cells, IBMX and ascorbic acid (100μM) plus or minus isoproterenol (100μM) were added to the medium. Ascorbic acid was added to prevent oxidation of isoproterenol. Six, 9 or 18 hours later, the cells were fixed and stained with 0.2mg/ml X-gal. Unfortunately, the number of positive cells observed following treatment with isoproterenol and IBMX was much smaller than the number seen following treatment with forskolin and IBMX. It was difficult to distinguish plates of cells treated with isoproterenol and IBMX from those exposed to IBMX alone.

Therefore, a quantitative assay was developed for the cAMP-mediated increase in β-galactosidase activity. This assay was performed in 96 well plates after solubilization of the cells and incubation with the substrate ONPG. Thus, the change in optical density over a defined period of time was directly proportional to the

level of β-galactosidase activity. The optical density increased linearly with time up to 1.5. The LVIP2.0Zc cells were determined to contain endogenous G_s-coupled receptors for PGE_2 (prostaglandin E_2) and adenosine. In pilot studies, untransfected cells were treated for 8 hours with IBMX in the presence or absence of NECA [5'-(N-ETHYLCARBOXAMIDO)-ADENOSINE, adenosine A_2 receptor agonist], or forskolin, to increase intracellular cAMP levels (Figure 1). NECA or forskolin-treated cells generated considerably higher levels of β-galactosidase activity than the IBMX-treated control cells. Similar results were obtained by incubations of cells with 1 or 10μM PGE_2. The enzyme levels generated by 1μM NECA, 10μM NECA, and 0.5μM forskolin were determined to be 8, 30, and 48-fold higher, respectively, than in the IBMX control cells. The differences were discernible by visual examination after 3 to 6 hours.

EXAMPLE 2

Induction of β-galactosidase: optimizing duration of exposure to ligands and doses of ligands.

In pilot studies, cells were exposed to IBMX in the presence or absence for forskolin (0.5μM), NECA (1μM), or prostaglandin E_2 (1μM) for 2, 4, 6, 8, 15, or 18 hours and their β-galactosidase activities measured. As shown in Figure 2, the excellent signal to noise ratios were obtained with drug/ligand treatments of 6 to 15 hours. Dose-response data (Figure 3) were obtained by treating cells with forskolin, NECA, or PGE_2 for 8 hours. Subsequent studies have shown that 6-8 hour exposures to ligand are optimal in

both the G_i and G_s assays. Th ref r , A 6-8 h ur drug tr atm nt was used; this way, the assay can be performed in ne day and th signal t nois ratio is quit good. Forskolin produced its maximum eff ct at a 0.5μM c ncentrati n; 10μM prostaglandin E_2 and NECA were required to achieve maximum enzyme induction.

EXAMPLE 3

Transfection of receptor cDNAs into LVIP2.OZc cells

The high efficiency transfection method of Chen and Okayama can be used to introduce plasmids containing receptor cDNAs into LVIP2.OZc cells. As previously shown, the amount of DNA used for transfection of cells is critically important: too little or too much DNA produces poor transient or stable expression of cDNAs. Five to 10μg of pcD plasmid containing β_2-adrenergic receptor cDNA were dissolved in the calcium/BES buffered saline transfection solution and added to the 10ml of tissue culture medium in 10 cm plates of cells. This amount of plasmid gave a punctuate precipitate which could readily be seen at 40x magnification and which gave the best induction of β-galactosidase in response to 1μM isoproterenol. The increase in β-galactosidase activity observed with IBMX in the presence or absence of isoproterenol was 2 to 4 times that observed with IBMX alone. The increase induced by isoproterenol was blocked by coincubation with the antagonist propranolol (1μM).

EXAMPLE 4

Induction of β-galactosidase in cells transfected with β_2-adrenergic receptor cDNA: optimizing the dose of isoproterenol and duration of exposure of cells to this compound

Three days after transfection with plasmids containing the β_2-adrenergic receptor cDNA, LVIP2.OZc cells were seeded in 96 well plates and incubated with isoproterenol, ascorbic acid and IBMX for 8 hours. The concentration of isoproterenol was varied from 10^{-12} to 10^{-4}M, a maximal effect was seen at 10^{-4}M; no further change in optical density was obtained for concentrations between 10^{-4}M; no further change in optical density was obtained for concentrations between 10^{-9} and 10^{-4}M. The EC_{50} value for isoproterenol was determined to be 10pM (Figure 4A). LVIP2.OZc cells were transfected as above, seeded in 96 well plates and incubated with 1μM isoproterenol, ascorbic acid and IBMX for 2, 4, 6, 8, 12 or 18 hours. The best signal to noise ratio was found after 6-8 hours exposure to drugs (Figure 4B).

EXAMPLE 5

Inhibition of forskolin-mediated induction of β-galactosidase by aldosterone secretion inhibitory factor (ASIF)

LVIP2.OZc cells were screened for endogenous receptors linked to G_i, which when activated would inhibit the increase in β-galactosidase activity mediated by treatment with forskolin. Among numerous agonists tested, only ASIF produced significant inhibition. ASIF was used to help optimize a screen for ligands which can interact with G_i-coupled receptors. Cells were

tr ated with IBMX, IBMX and forsk lin (0.5μM), r IBMX, forsk lin, and ASIF (1μM) for 1, 2, 4, 6, 8, 15, or 18 h urs. Th m st robust inhibition of forskolin by ASIF was bs rved betw en 6 and 8 hours (Figur 5). Low r concentrations of forskolin (0.1 to 0.5μM) should be used, however, to observe maximum inhibition in the assay. The highest dose of forskolin is not inhibited as well as the two lower doses, but the former may give a more reproducible level of enzyme induction.

EXAMPLE 6

Inhibition of forskolin-mediated induction of ß-galactosidase by dopamine or quinpirole following transfection of cells with dopamine receptor cDNA

Cells were transfected with 5, 10, 15, or 20 μg of D_{2A}Rc/RSV, a plasmid containing the D_{2A} receptor isoform. Ten μg of plasmid per 10 ml in a 10 cm dish gave the best looking precipitate, and aliquots of a suspension of cells from this dish were added to wells of a 96 well plate. Three days after the transfection, IBMX was added to all of the wells. ß-galactosidase activity was elevated with forskolin (0.5μM) alone or in combination with dopamine, or the D_2 selective agonist, quinpirole, to block this increase. The dopaminergic agonists were added in concentrations of 1 to 100μM. 100 μM ascorbic acid was added to the medium to prevent oxidation of the ligands (Figure 6A). Dopaminergic agonists alone had little or no effect on ß-galactosidase levels. Significant inhibition of forskolin-stimulated increase of ß-galactosidase activity was observed with either 10μM dopamine or 10 or 100μM

quinpirole. As expected, quinpirole was more potent than dopamine.

Transfections and assays as described above were used successfully to "diagnose" the rat cannabinoid or the human muscarinic m_2 receptor. Addition of 1μM agonist CP55940 decreased forskolin stimulated activity by 15% in cells expressing the cannabinoid receptor (Figure 6B). Similar decrease was observed after addition of 1mM carbachol to cells expressing the muscarinic acetylcholine receptor (Figure 6C).

Table 1

SCREENING PROTOCOL

Day 1	Trypzinize xponentially growing LVIP2.OZc cells, seed 5x10^5 cells/10 cm-plate, incubate overnight in 10ml growth medium.
Day 2	Transfect cells with 10-20μg plasmid DNA using Ca_3PO_4 technique, see Materials and Methods, incubate 15 to 24 hours at 35°C under 2-4% CO_2.
Day 3	Wash cells 2x with growth medium, refeed, incubate 24 hours at 37°C under 5% CO_2.
Day 4	Trypzinize cells, pellet, seed in 96 well microtiter plates 5-10x10^4 cells/well, incubate overnight in 100μl growth medium.
Day 5	To control wells (e.g, quadruplicates) add 100μl medium + 1mM IBMX with (control stimulation) or without (control basal) 1μM forskolin. To remaining wells add agonist(s) in 100μl medium + 1mM IBMX with (G_i-coupled), or without (G_s-coupled) 1μM forskolin. Include antioxidants and/or protease inhibitors as required. Incubate 6-8 hours at 37°C under 5% CO_2. Wash cells with 200μl PBS and drain. (Cells can be stored at -20°C for 24-72 hours).
Day 6	Add 25μl diluted assay buffer/well, wait 10 minutes, then add 100μl assay buffer, wait 10 minutes, then add 25μl of substrate. (ONPG 4mg/ml assay buffer). View color development visually or measure spectrophotometrically at 405nm in a plate reader.

* * * *

All publications mentioned hereinabov are hereby incorp rat d in their ntirety by r ferenc .

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

WHAT IS CLAIMED IS:

1. A method of identifying ligands which bind to G-protein coupled receptors comprising:

expressing a G_i-coupled receptor gene in a eucaryotic cell wherein said cell contains a cyclic AMP sensitive reporter construct;

adding a ligand to said cell; and

assaying for the amount of cyclic AMP.

2. The method according to claim 1, wherein said cyclic AMP sensitive reporter construct comprises a cyclic AMP responsive enhancer element.

3. The method according to claim 2, wherein said cyclic AMP sensitive reporter construct further comprises a Lac Z gene.

4. The method according to claim 3, wherein said cyclic AMP sensitive reporter construct is pLVIP2.OZ.

5. The method according to claim 4, wherein said cell comprises a cell from cell line LVIP2.OZc.

6. The method according to claim 1, wherein said assaying step comprises adding a chromogenic substrate and assaying for change in chromogenic substrate.

7. The method according to claim 6, wherein said chromogenic substrate is o-nitrophenyl ß-D-galactopyranoside.

8. The method according to claim 1, wherein said G-coupled receptor gene is encoded by a cDNA.

9. A method of identifying an antagonist of a ligand wherein said ligand binds to a G-protein coupled receptor comprising:

expressing a G-coupled receptor gene or cDNA in a eucaryotic cell wherein said cell contains a cyclic AMP sensitive reporter construct;

adding a ligand and antagonist to said cell; and

assaying for the amount of cyclic AMP.

10. The method according to claim 9, wherein said cyclic AMP sensitive reporter construct comprises a cyclic AMP responsive enhancer element.

11. The method according to claim 9, wherein said cyclic AMP sensitive reporter construct further comprises a Lac Z gene.

12. The method according to claim 11, wherein said cyclic AMP sensitive reporter construct is pLVIP2.OZ.

13. The method according to claim 12, wherein said cell comprises a cell from cell line LVIP2.OZc.

14. The method according to claim 9, wherein said assaying step comprises adding a chromogenic substrate and assaying for change in chromogenic substrate.

15. The method according to claim 14, wherein said chromogenic substrate is o-nitrophenyl ß-D-galactopyranoside.

16. The method according to claim 9, wherein said G-coupled receptor gene is a G_i-coupled receptor gene.

17. A method of identifying ligands which bind to a G-protein coupled receptor comprising:

expressing a G_i-coupled receptor gene of cDNA in a eucaryotic cell wherein said cell contains a cyclic AMP sensitive reporter construct;

adding forskolin and a ligand;

assaying for the amount of cyclic AMP; and

identifying agonist induced reduction of forskolin activity.

18. A method of identifying an antagonist of a ligand which binds to a G-protein coupled receptor comprising:

expressing a G_i-coupled receptor gene of cDNA in a eucaryotic cell wherein said cell contains a cyclic AMP sensitive reporter construct;

adding forskolin, ligand, and antagonist;

assaying for the amount of cyclic AMP; and

identifying agonist induced reduction of forskolin activity.

19. A mouse L cell that comprises a recombinant cyclic AMP sensitive reporter construct.

20. The cell according t claim 19, wherein said construct compris s a cyclic AMP r sponsiv nhanc r lement.

21. The cell acc rding t claim 19, wherein said cyclic AMP sensitive reporter construct comprises a Lac Z gene.

22. The cell according to claim 19, wherein said construct is stably integrated into genomic DNA of said cell.

23. The cell according to claim 19, wherein said construct is pLVIP2.OZ.

24. The cell according to claim 23, wherein said cell is a LVIP2.OZc cell.

FIG. 1

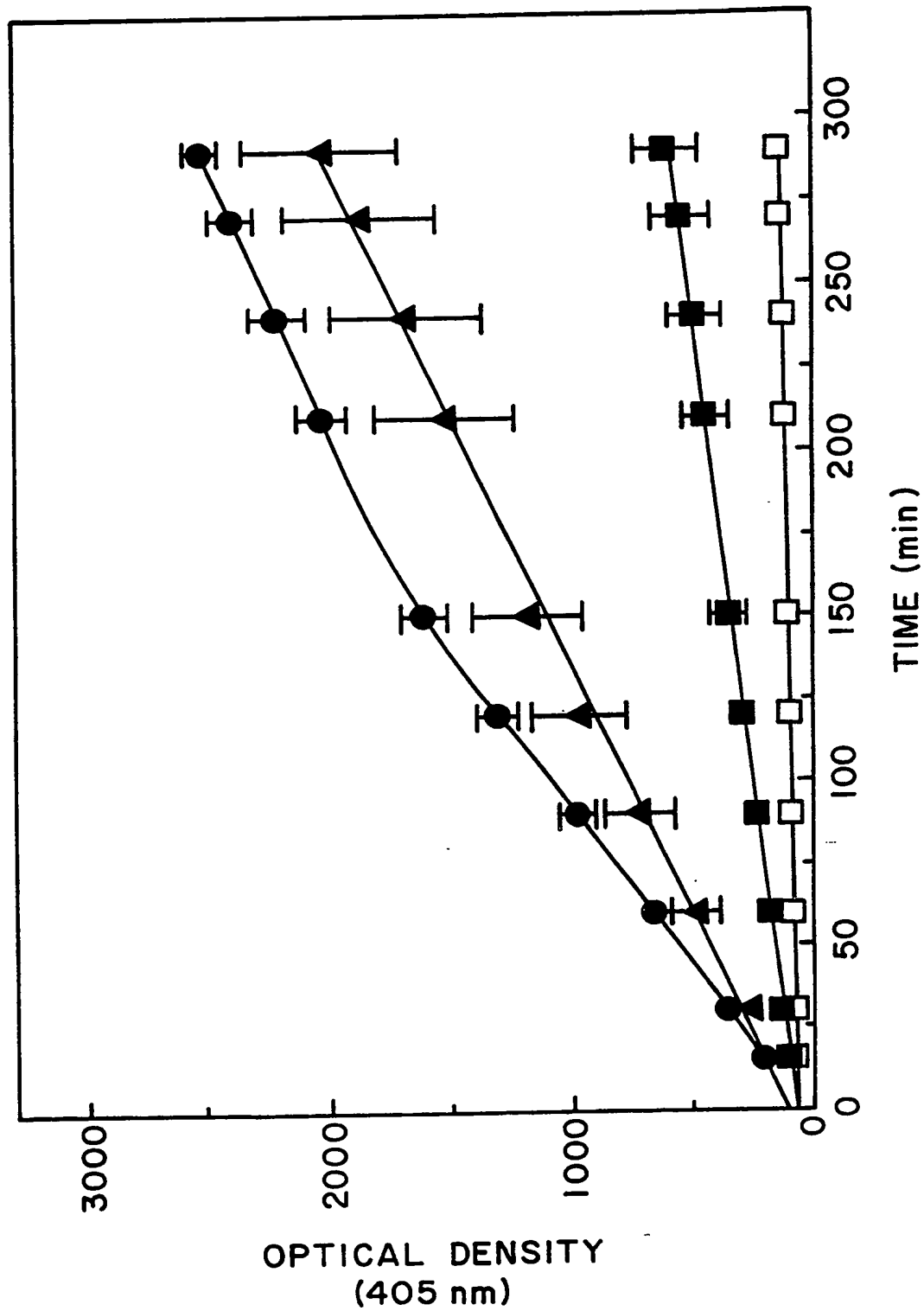

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FIG. 2

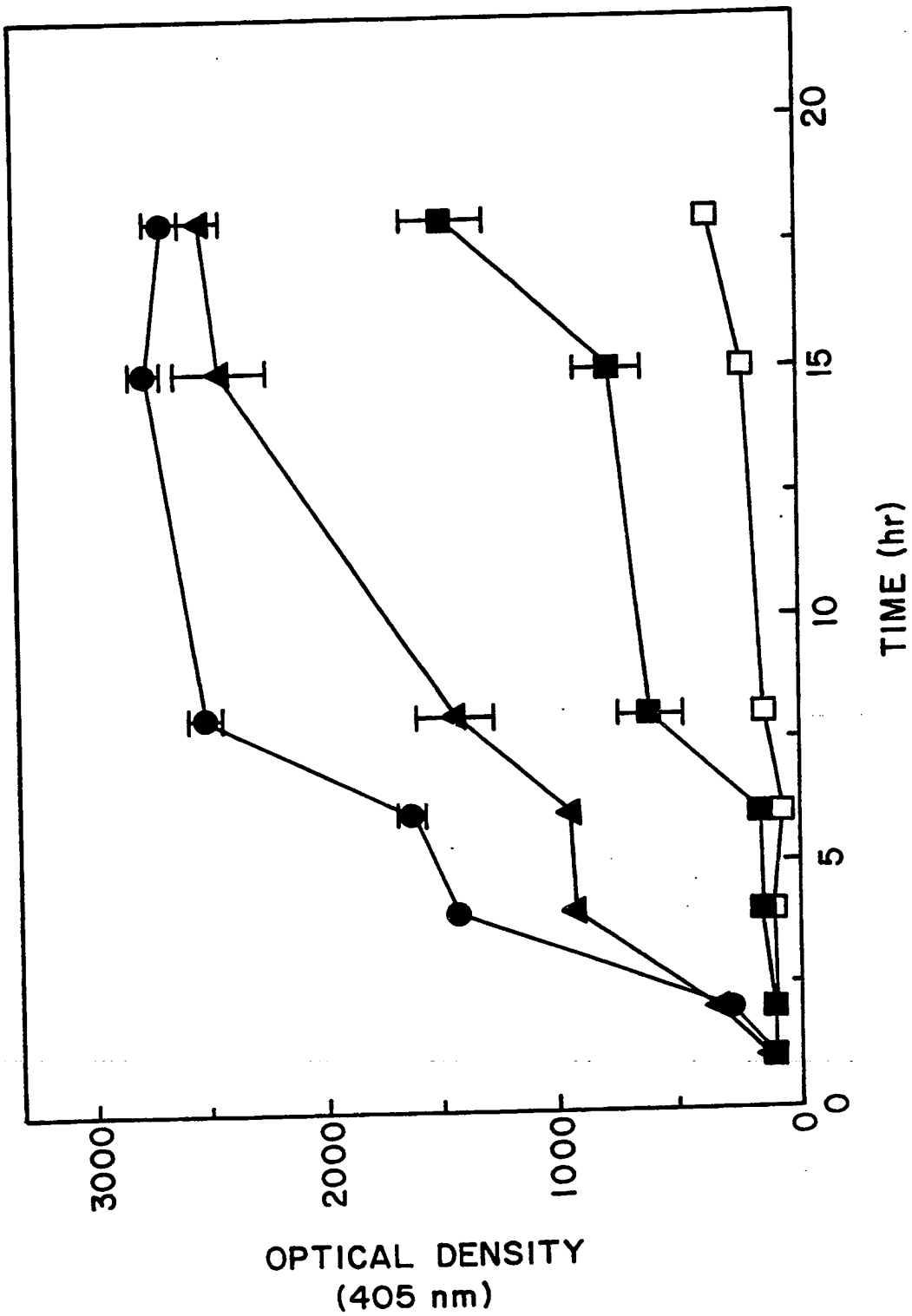

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FIG. 3

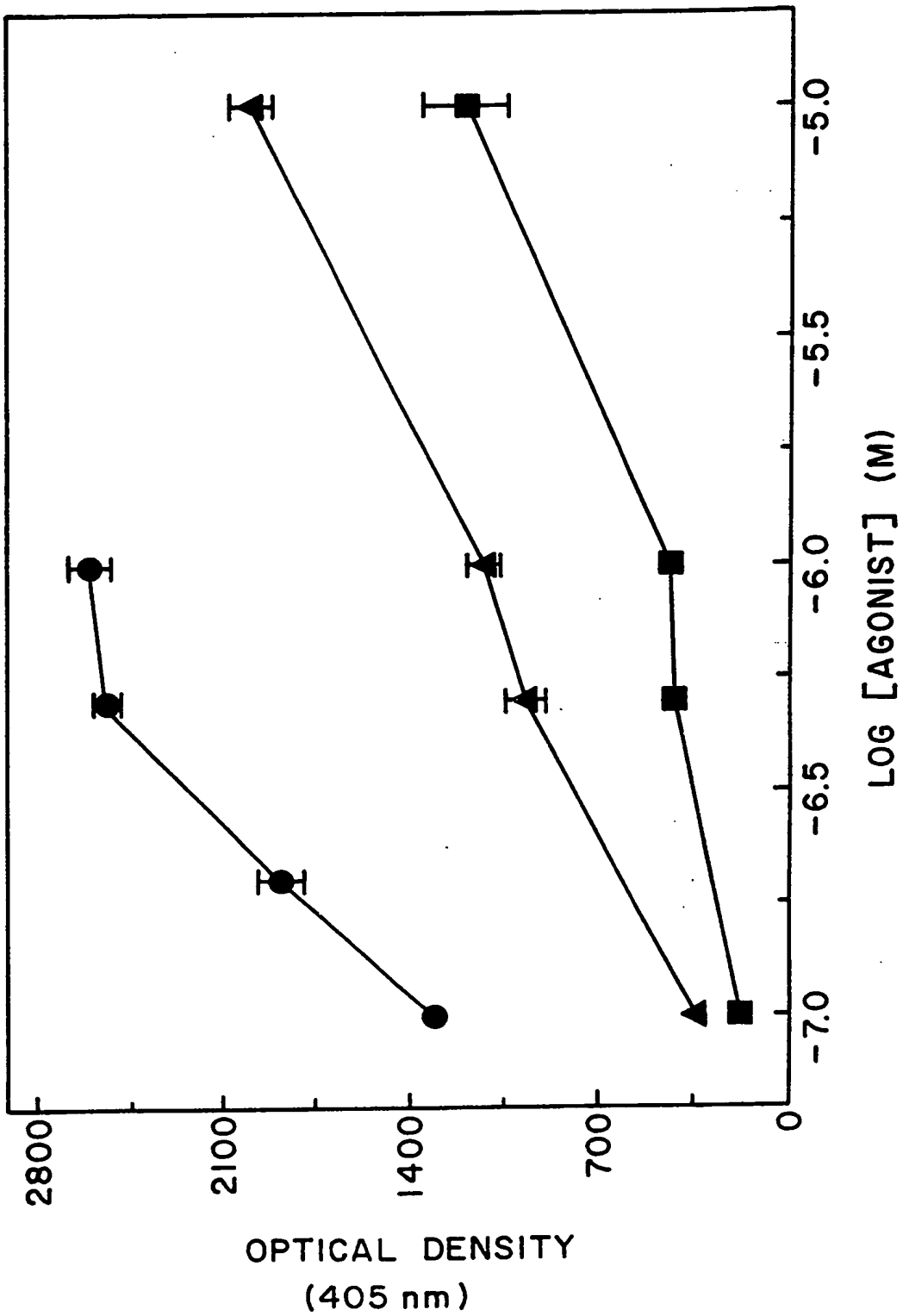

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FIG. 4a

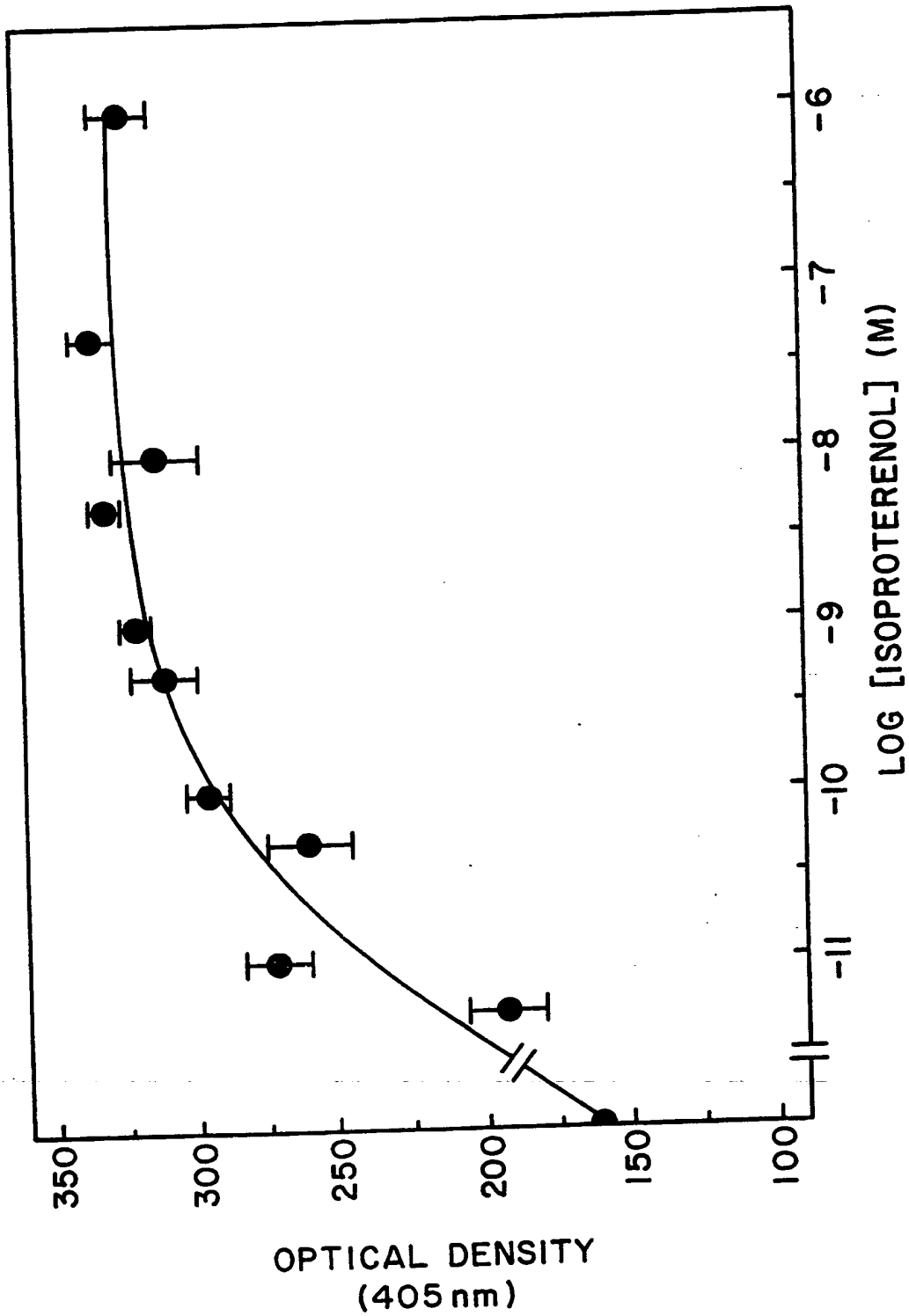

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FIG. 4b

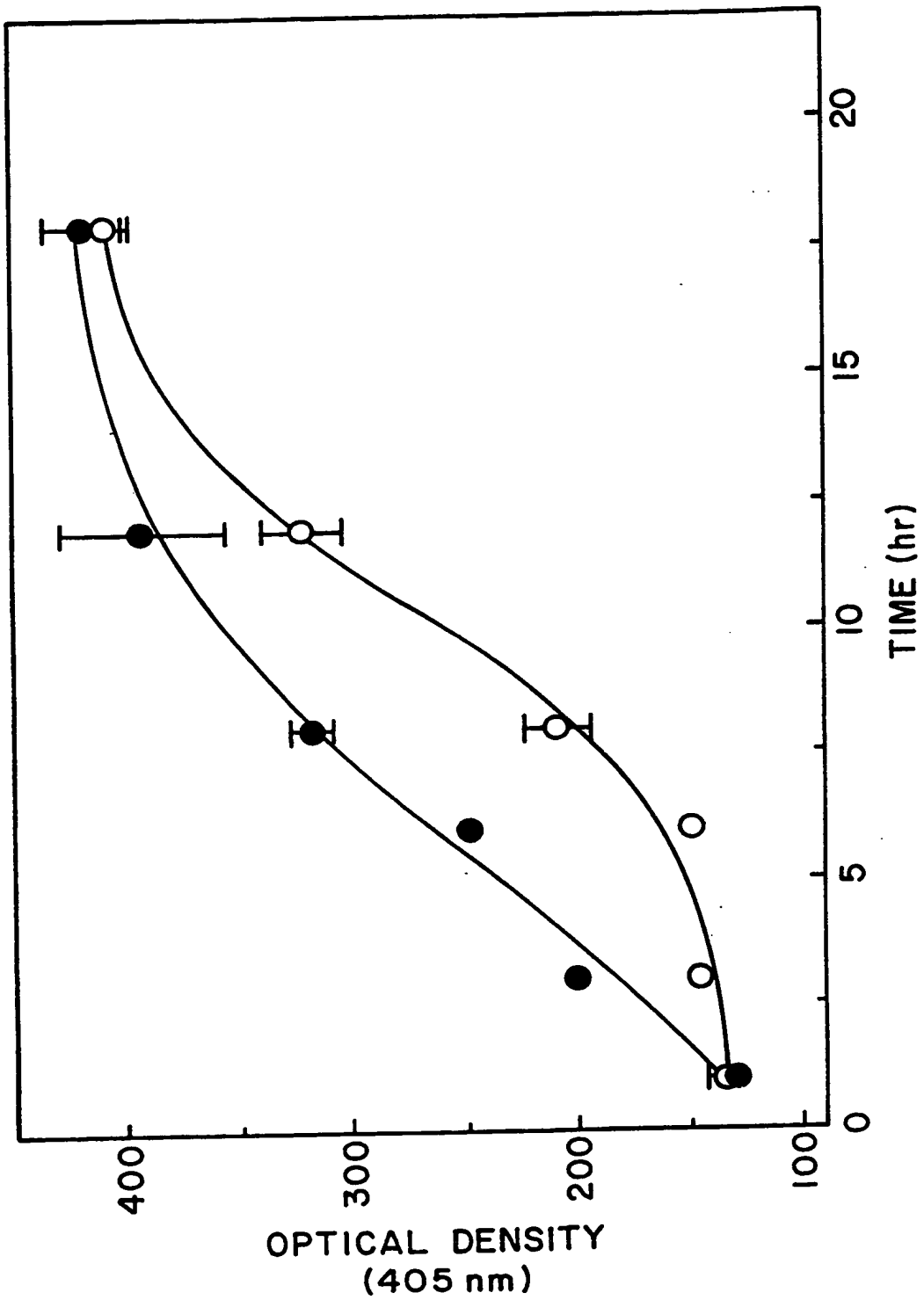

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FIG. 5

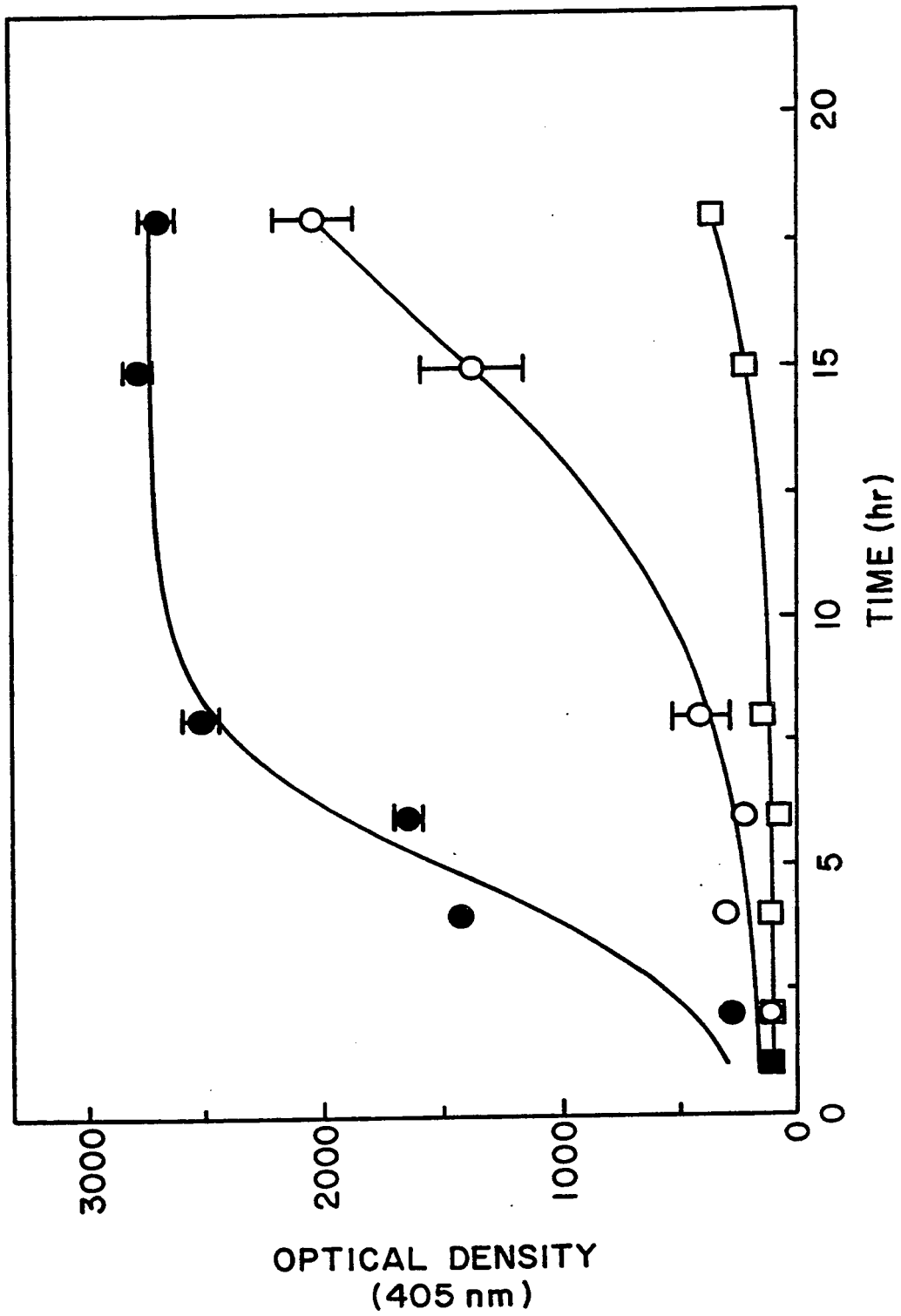


FIG. 6b

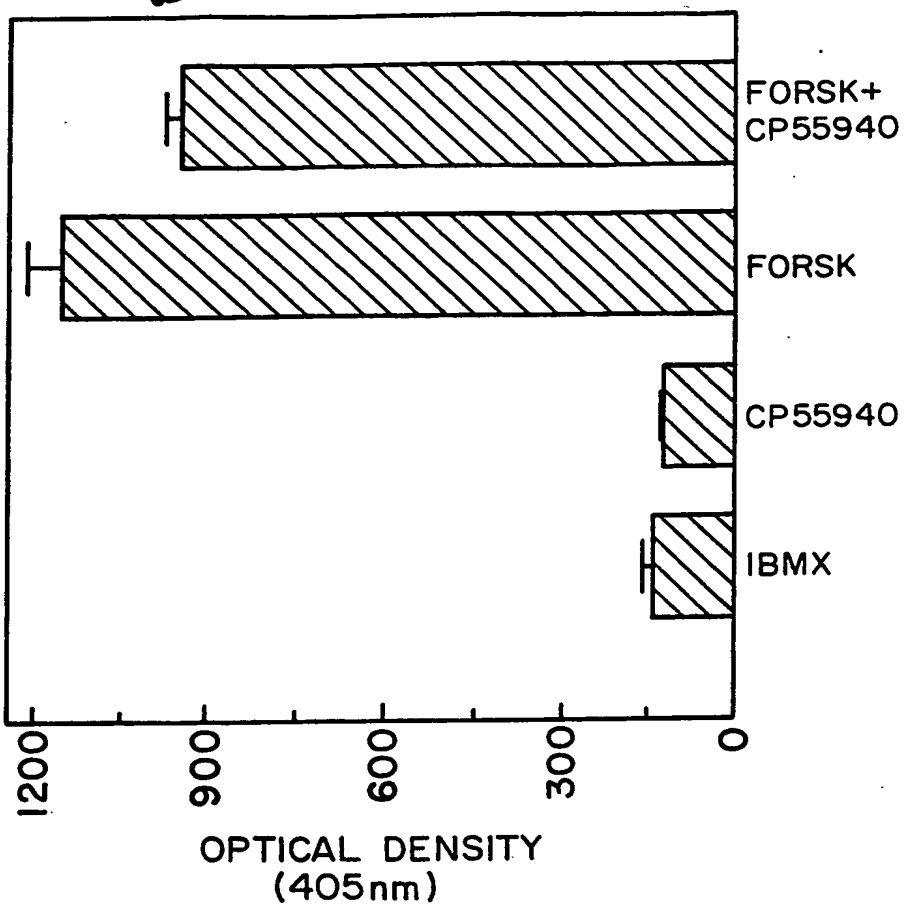
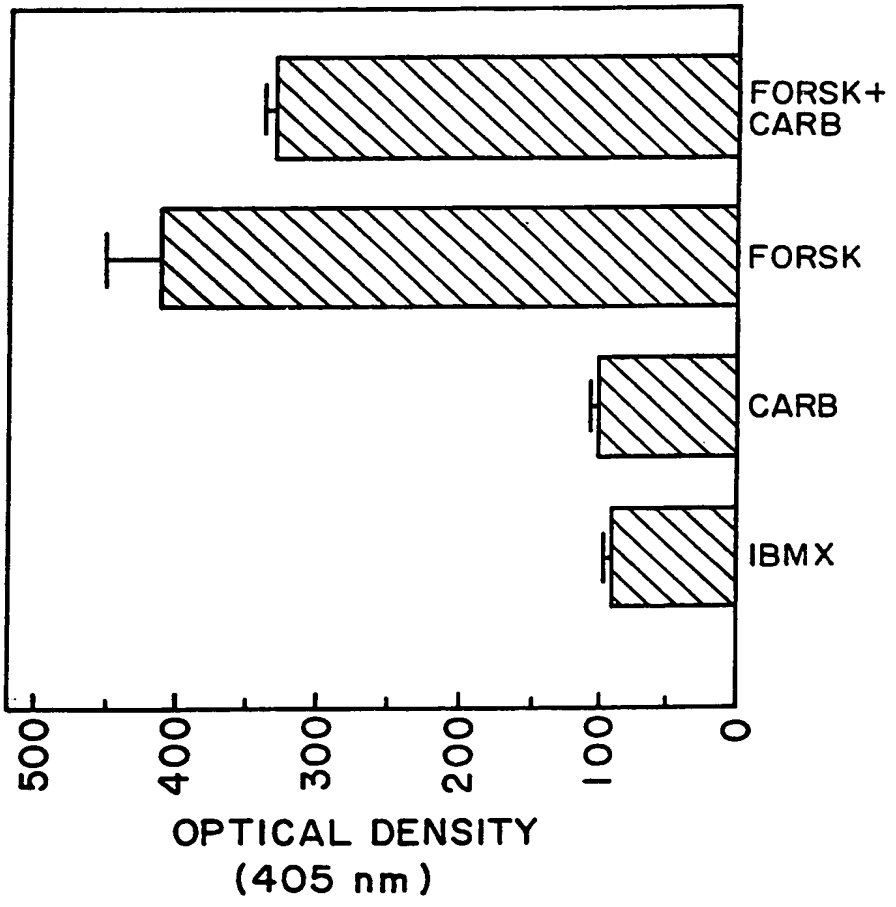


FIG. 6a



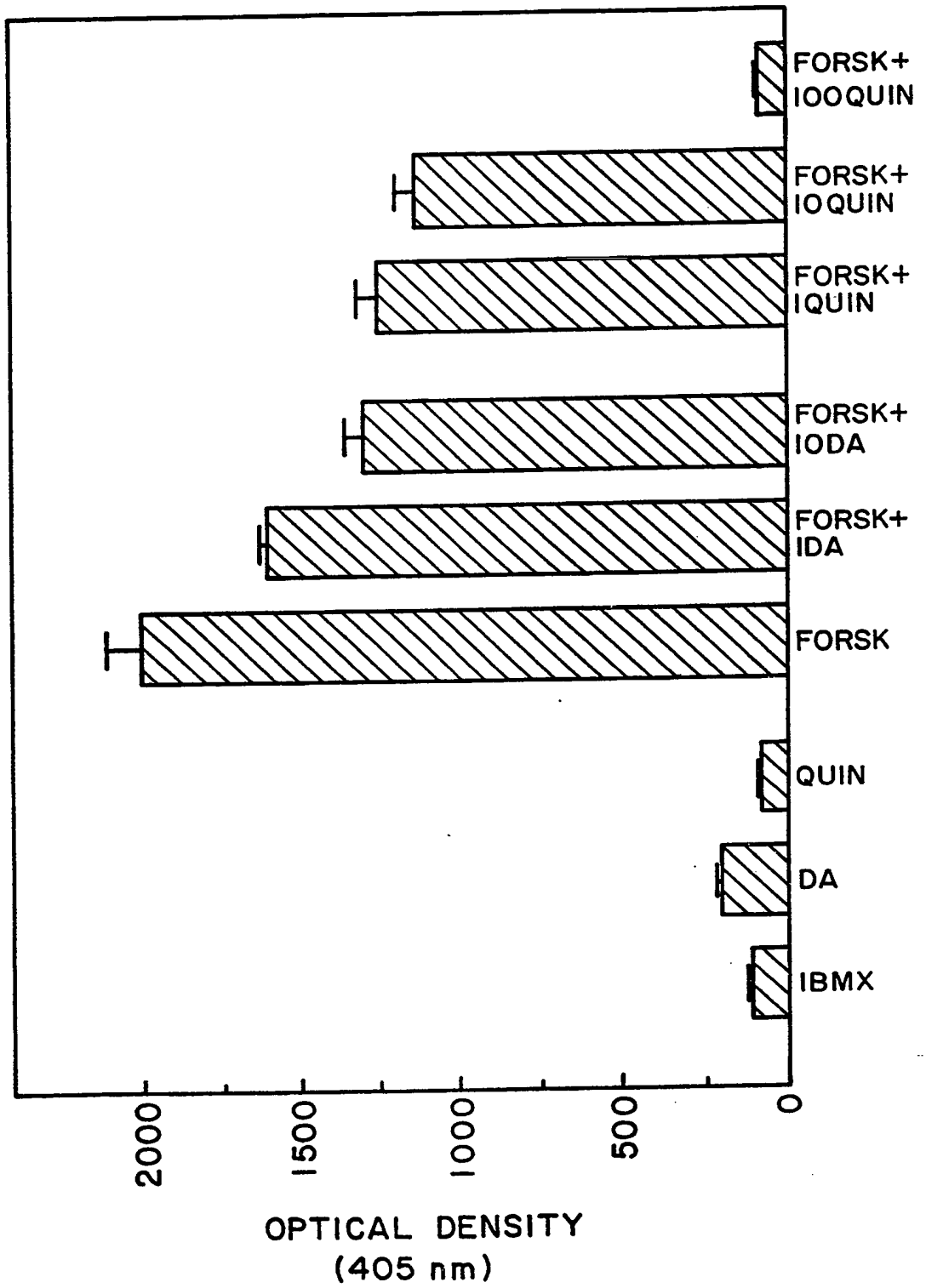


FIG. 6c

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INTERNATIONAL SEARCH REPORT

International application No. PCT/US92/08202

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68, 1/00; G01N 33/53; C12P 21/06; C12N 5/00
US CL :435/6, 7.1, 7.23, 69.1, 240.1, 240.2, 240.27; 935/34, 60, 66, 70
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 7.23, 69.1, 240.1, 240.2, 240.27; 935/34, 60, 66, 70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, VOLUME 336, ISSUED 03 NOVEMBER 1988, RIABOWOL, "THE CATALYTIC SUBUNIT OF CAMP-DEPENDENT PROTEIN KINASE INDUCES EXPRESSION OF GENES CONTAINING CAMP-RESPONSIVE ENHANCER ELEMENTS", PAGES 83-86, ENTIRE DOCUMENT.	1-24
Y,P	US, A, 5,126,251 (MOSS ET AL) 30 JUNE 1992, CLAIMS 1-13, ENTIRE DOCUMENT.	1-24

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 24 OCTOBER 1992	Date of mailing of the international search report 08 JAN 1993
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer GIAN WANG Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*